Use of thermographic imaging to screen for drought-tolerant genotypes in *Brachypodium distachyon*

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Abstract. Thermal imaging has been used to evaluate the response to drought and warm temperatures in a collection of *Brachypodium distachyon* lines adapted to varied environmental conditions. Thermographic records were able to separate lines from contrasting rainfall regimes. Genotypes from dryer environments showed warmer leaves under water deficit, which suggested that decreased evapotranspiration was related to a more intense stomatal closure. When irrigated and under high temperature conditions, drought-adapted lines showed cooler leaves than lines from wetter zones. The consistent, inverse thermographic response of lines to water stress and heat validates the reliability of this method to assess drought tolerance in this model cereal. It additionally supports the hypothesis that stomatal-based mechanisms are involved in natural variation for drought tolerance in *Brachypodium*. The study further suggests that these mechanisms are not constitutive but likely related to a more efficient closing response to avoid dehydration in adapted genotypes. Higher leaf temperature under water deficit seems a dependable criterion of drought tolerance, not only in *B. distachyon* but also in the main cereal crops and related grasses where thermography can facilitate high-throughput preliminary screening of tolerant materials.

Additional keywords: abiotic stresses, high-throughput phenotyping, water deficit.

Introduction

In many regions of the world, abiotic stresses, mostly water and temperature, limit current cereal yields and the genetic gains derived from plant breeding (Araus *et al.* 2002). Droughts and heat waves are expected to be more frequent with climate change (Intergovernmental Panel on Climate Change 2013); therefore, a challenge in plant breeding is the development of new crop varieties that maintain good and steady yields under unfavourable abiotic conditions. This goal is hampered by the complex genetic basis of plant adaptation to drought and high temperatures. Identification of genes involved in adaptive response to those stresses, and development of molecular markers for assisted selection in segregant progenies, are primary goals.

Stomatal regulation is one of the mechanisms that plants have evolved to face climate constraints. In most cases, the plant's first response to low water availability is to maintain water content close to the unstressed level by stomatal closure. The consequent decline in transpiration causes an increase in leaf temperature (LT), which can be monitored to estimate stomatal response (Verslues *et al.* 2006). Rapid stomatal response to changes in soil water potential is a well-known mechanism in plants adapted to drought conditions (Davies *et al.* 2005). Conversely, a less sensitive response characterises plants from wetter environments, where adaptation to drying soil is less crucial to ensuring productivity. In plants with unlimited access to water, high temperatures induce stomatal aperture, as has been directly shown by microscopy image analyses (Feller 2006; Reynolds-Henne *et al.* 2010). Plants adapted to heat stress show increased transpiration when water is available. This induces leaf cooling through evaporation of water associated with latent heat lost (Gupta *et al.* 2012). Therefore, the LT of plants with unlimited access to water might be used to characterise stomatal opening and genetic variation in response to heat stress.

Thermographic approaches do not measure leaf transpiration, but the linear relationship between LT change and variation in stomatal conductance or aperture is well documented (e.g. Hashimoto *et al.* 1984; Jones 1999; Kümmerlen *et al.* 1999; Grant *et al.* 2007). Thermal imaging has been established as a suitable method of high-throughput phenotyping to detect differences in stomatal conductance in response to heat or water

stress (Merlot *et al.* 2002; Munns *et al.* 2010). Thermographic cameras are accurate and precise devices to monitor LT, so they might be a useful tool to characterise genetic variation related to drought and heat adaptation in large germplasm collections.

The easy handling, small genome and increasing number of analytical tools available for model plants make them a useful resource for the molecular dissection of agronomically important traits in crops with large complex genomes such as wheat (16 000 Mb). The transferability of such knowledge from the model to the crop species requires a high degree of synteny and colinearity between their genomes. In addition, the growth conditions and demands of the model must be similar to those of the crop when traits under study are related to plant response to external agents such as water availability and temperature. These are the main reasons why the monocot annual grass *Brachypodium distachyon* (272 Mb) has been recognised, over *Arabidopsis* and rice, as the most suitable model plant for functional genomics of temperate grasses and cereals of economic relevance, such as wheat and barley (Draper *et al.* 2001; Catalan *et al.* 2014). Luo *et al.* (2011) demonstrated natural variation for drought response in *B. distachyon* by measuring a set of physiological and biochemical parameters that are laborious and require specialised equipment.

The present study was conducted on lines of *B. distachyon* developed from wild plants collected at sites with contrasting rainfall and temperature conditions within the Iberian Peninsula. Our main aim was to search for genotypes with an efficient, stomatal-based response to drought by using thermography. The relation between the climatic characteristics of the native location of the lines and their thermal response to water deficit and warm temperatures has been further explored to validate the reliability of this simple approach for fast screening of drought-tolerant genotypes.

Materials and methods

Plant material

Sixty-one lines of *B. distachyon* (2n = 2x = 10) were used in the analysis. Each line derives from a specimen collected in the wild. The full set of lines represents 32 locations quite evenly distributed from the north to south of Spain and ranging from 357 to 1525 m in elevation above sea level. The collection sites, mostly representing mountain and continental climate zones, were usually on high plateaus or in mountain valleys or slopes. Detailed information on the geographical location and main climatic parameters of the collecting sites is given in the Supplementary Material, table A (available at the Journal's website). Genotypic differences between lines derived from distinct plants collected at the same location had been detected in most of these *B. distachyon* populations, based upon molecular profiling with 15 microsatellite (simple sequence repeat, SSR) markers conducted by Giraldo *et al.* (2012). For each of the 29 polymorphic populations found, two of the most divergent lines according to their SSR profiles were selected for the present study. To keep some of that genetic variability represented in the analyses, lines from the same population have been independently considered.

The reference line Bd21, whose sequencing has been completed (International Brachypodium Initiative 2010), was examined in the study but it was excluded from statistical analysis because information on the bioclimatic variables for its location of origin was unavailable.

Experimental design

Four blocks of lines were established. For each block, the 61 lines were individually planted in plastic pots 10 cm by 10 cm and randomly distributed in a tray 1 m by 1 m. At the onset of the tillering stage, treatment plants (three blocks) received no irrigation, whereas their control replicates (one block) were maintained in well-watered condition throughout the experiment. This experimental design, with one single irrigated replicate of each line, seemed appropriate because the standard deviation of canopy temperature under full water availability conditions at the root-zone is virtually 0 (e. g. González-Dugo *et al.* 2006). The plants were grown for the whole experiment in a greenhouse equipped with an inner sunshade curtain and a cooling system that operates when the indoor air temperature reaches 27°C.

Thermographic images of treated and irrigated control plants were taken with a FLIR SC305 camera (Inframetrics; FLIR Systems Inc., Wilsonville, OR, US). The camera has a resolution of 320 by 240 pixels and thermal sensibility of 0.01°C from −20°C to +120°C. Images were taken from a zenithal position at 1.2 m height over the pots. Images were visualised with the software ThermaCAM Researcher Professional 2.10 (FLIR Systems Inc.) and saved as Excel files in which a temperature was recorded for each single pixel. The mean leaf temperature of stressed (LTs) and control (LTc) plants was estimated based on the thermographic records from selected pixels (183 per image) that strictly corresponded to leaf tissue. Simultaneously, visual colour images were taken with a digital camera (IXUS 100IS; Canon Inc., Tokyo) to evaluate the wilting symptoms (Fig. 1).

Images were taken at 12 : 00 after 2 weeks (LTs1 and LTc1 values) and 3 weeks (LTs2 and LTc2 values) of drought treatment, with indoor air temperatures of 19°C and 28°C, respectively, and outdoor temperature–sun radiation of 13.5°C–630 W m^{-2} and 27.5°C–740 W m^{-2}, respectively. Based on LT estimations, the thermal difference (TD) between leaves of water-stressed and control plants was calculated for each recording date: variables TD1 (LTs1 – LTc1) and TD2 (LTs2 – LTc2).

Each experimental block included three non-sown, soil-filled pots, which were also thermographed to estimate soil temperatures (ST), considered as providing a useful estimate of the micro-ambient temperature affecting stressed and irrigated plants.

Climatic variables

Table 1 shows the climatic variables included in this study. For some of them, records used in the analyses were directly taken from Gonzalo (2010), who authored a climate model for functional classification of Spanish vegetation and Peninsular ecosystems. In that model, which has a resolution of 30 arc-seconds, temperature and precipitation values are interpolated by using ordinary co-kriging, with altitude, distance to the coast, potential sun radiation and cloudiness as secondary variables to estimate the monthly climatic variables. Thus, the resulting regionalisation is very precise for the Iberian Peninsula.

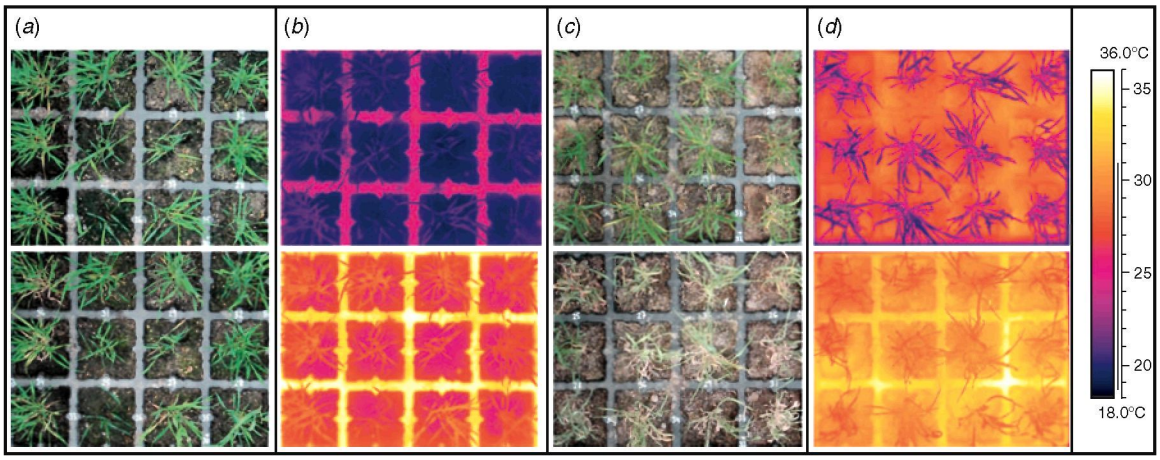

Fig. 1. Visual and thermographic images of (*a*, *b*) irrigated control and (*c*, *d*) stressed plants of 12 of the *B. distachyon* lines after 2 weeks (upper images) and 3 weeks (lower images) of drought treatment. Colour code of measured temperatures is included.

Table 1. Climatic variables included in the study

Variable	Abbreviation
Monthly mean temperature (January–June)	TMEA_1 to TMEA_6
Monthly minimum temperature (January–June)	TMIN_1 to TMIN_6
Monthly maximum temperature (January–June)	TMAX_1 to TMAX_6
Lowest monthly mean temperature	TMEA_LOW
Annual mean temperature	TMEA_ANN
Annual minimum temperature	TMIN_ANN
Annual maximum temperature	TMAX_ANN
Mean temperature of wettest quarter[A]	TMEA_WET_Q
Mean temperature of coldest quarter[A]	TMEA_COL_Q
Minimum temperature of coldest month[A]	TMIN_COL_M
Temperature seasonality[A]	T_SEA
Isothermality[A]	T_ISO
Mean diurnal temperature range[A]	TMEA_DIU_R
Annual temperature range[A]	T_ANN_R
Monthly precipitation (January–June)	PREC_1 to PREC_6
Annual precipitation	PREC_ANN
Precipitation of wettest quarter[A]	PREC_WET_Q
Precipitation of coldest quarter[A]	PREC_COL_Q
Precipitation of wettest month[A]	PREC_WET_M
Precipitation seasonality[A]	PREC_SEA
Lang aridity index[A]	LANG

[A]Values calculated from data in Gonzalo (2010).

Besides raw climatic variables (monthly or annual precipitations and temperatures), our study has also considered some bioclimatic variables (www.worldclim.org/bioclim) whose values for each collecting site were derived from the temperature and rainfall data in Gonzalo (2010). The aridity index of Lang (Lang 1920) was also estimated from the annual mean temperature and precipitation values of Gonzalo (2010). In the Iberian Peninsula, *Brachypodium distachyon* has a short life cycle; flowering occurs during spring, and maturation and shedding during early summer. The period July–December has little or no effect on the growth of the plant. Thus, we have not considered in the analysis the monthly mean temperatures or precipitation for that period, or the bioclimatic variables based on those climatic data (e.g. precipitation of the driest quarter).

Statistical analyses

Analyses of variance (ANOVA) were performed to determine the effects of genotype (or line) and location of origin of the line (equivalent to 'locality' in Supplementary table A) on the thermographic variables, and to evaluate the influence on the thermographic variables of climate characteristics of the sites where the collected populations were found. Stepwise regression analyses were conducted with the thermographic variables as dependent variables and the climatic data of the site of origin of lines as independent variables. Differences between means and relations between variables were tested using Student's *t*-test and Pearson correlation coefficients, respectively. Cluster analysis and dendrograms were performed using the unweighted pair group with arithmetic averaging (UPGMA) aggregation method with the Euclidean distance as a similarity measure. Statistical analyses were conducted with SPSS version 8.0, 1998 (SPSS Inc., Chicago, IL, USA) and Infostat (Di Rienzo *et al.* 2014) statistical packages.

Results

Thermographic images were able to distinguish between ST and LT in the water-stressed and control (irrigated) plants (Fig. 1). No wilting was observed in control plants throughout the experiment (Fig. 1*a*). The thermographic images indicated that leaves were usually warmer than the irrigated background soils and that LT and ST were higher at the second recording date than the first (Fig. 1*b*). After 2 weeks of drought treatment, stressed plants showed medium wilting symptoms in the visual colour image and maintained LT values clearly below ST (Fig. 1*c*, *d*, upper images). After 3 weeks, water-stressed plants showed extreme wilting symptoms in the visual colour image, and the thermographic images still allowed distinction between leaves and the background ST (Fig. 1*c*, *d*, lower images).

Based on the thermographic images of non-sown, soil-filled pots, the mean temperature values estimated the first recording date for irrigated and non-irrigated soils were 20°C (STc1) and 25°C (STs1), respectively. One week later, values rose to 28°C and 35°C (STc2 and STs2, respectively). The ST increment was

Table 2. Descriptive statistics of the leaf temperatures (LT) estimated under control (watered) conditions and drought stress, and derived thermal differences (TD) in the set of 61 *Brachypodium distachyon* lines

ANOVA results on the significance of the effects of genotype (line) and locality of origin of the lines on the LT variables are also included

Thermographic variable	Abbreviation	Mean	Minimum	Maximum	Standard deviation	Coefficient of variation	Source of variation	
							Line	Locality
LT of irrigated control plants, 1st recording date	LTc1	20.47	18.64	23.92	0.95	4.63		$P=0.005$
LT of irrigated control plants, 2nd recording date	LTc2	28.96	27.44	30.56	0.79	2.74		$P<0.001$
LT of drought-stressed plants, 1st recording date	LTs1	22.54	20.95	24.29	0.91	4.05	$P=0.2608$	$P<0.001$
LT of drought-stressed plants, 2nd recording date	LTs2	30.15	28.71	31.64	0.70	2.33	$P=0.0467$	$P<0.001$
LTs1 – LTc1	TD1	2.09	–1.97	5.41	1.49	71.01	$P<0.001$	$P<0.001$
LTs2 – LTc2	TD2	1.19	–1.67	3.21	1.27	106.18	$P<0.001$	$P<0.001$

clearly a reflection of the warmer outdoor temperatures experienced at the second recording date. These values seemed useful to assess differential micro-ambient temperature conditions affecting drought-stressed and control blocks.

Table 2 presents a basic statistical description of the thermographic variables analysed in the 61 lines (detailed data for each line are given in Supplementary Material table B). The leaf temperature of watered plants was based on thermographic images of one plant per line, taking into consideration that the standard deviation of canopy temperature has been found to be close to 0 when measured in plots of fully irrigated plants (Gardner *et al.* 1981; see also González-Dugo *et al.* 2006). LTc values ranged around a mean slightly higher than the corresponding STc value at any of the two recording dates (20.5°C for LTc1 and 29.0°C for LTc2). The mean LT values of stressed plants were ~3–4°C below estimates of STs (22.5°C for LTs1 and 30.2°C for LTs2), although some lines showed even greater differences (see minimum LTs values in Table 2).

The LTs values were mostly higher than LTc values at both dates of image recording, which is indicated by TD1 and TD2 mean values >0. However, a few lines showed negative thermal difference between drought and irrigated conditions. This was observed in 2 and 11 lines for TD1 and TD2, respectively (Supplementary table B). The narrower range of TD2 values indicated that differences were less pronounced at the end of the experiment (Table 2). Under irrigated conditions, the average difference in LT between recording dates was similar to the wet ST increment (LTc2 – LTc1 mean = +8.5°C). Such a noticeable increase can be ascribed to the much warmer conditions during the latter thermography session, with an outdoor temperature 14°C higher than recorded during the first session. The range of LTc2 values indicated that some lines were able to maintain leaf temperatures as much as 3°C below others.

For the ANOVA, in the case of variables LTc1 and LTc2, data are based on one plant per line, and then, only the effect of locality could be analysed. Results of the ANOVA (Table 2) indicated no effect or only slight effect of genotype on LTs variables, but a highly significant effect on TD variables. In turn, the locality of origin was found to be a highly significant source of variation of LT values in control and water-stressed plants at the two recording dates, as well as in derived TD values (Table 2).

Correlation analyses were performed between thermographic variables (Table 3). No relation was found between LT values recorded in control plants during experiment (LTc1 *v.* LTc2). However, the LT values of plants after 2 and 3 weeks of drought treatment (LTs1 and LTs2) showed a highly significant, positive correlation, evidencing a consistent behaviour of the lines under medium to high water-stress conditions. Negative correlation coefficients between LT values recorded in control and water-stressed blocks during the same thermographic session (LTc1 *v.* LTs1, LTc2 *v.* LTs2; Table 3) indicated that under similar indoor temperature conditions, lines with cooler leaves in irrigated conditions showed warmer leaves when water stressed. The negative significant correlation between LTc2 and LTs1 provided evidence that lines showing the highest LT with medium water stress had the lowest LT when watered but in warm conditions. Finally, the TD values recorded after 2 and 3 weeks of water deficit (TD1 *v.* TD2) were positively correlated (Table 3).

Table 3. Correlation coefficients between the thermographic variables analysed

For description of the thermographic variables, see Table 2. *$P<0.05$; **$P<0.01$

Thermographic variables	*r* value
LTc1 *v.* LTc2	0.17
LTs1 *v.* LTs2	0.63**
LTc1 *v.* LTs1	–0.27*
LTc2 *v.* LTs2	–0.42**
LTc1 *v.* LTs2	0.23
LTc2 *v.* LTs1	–0.58**
TD1 *v.* TD2	0.42**

Under the experiment conditions, it can be assumed that LTs2 values were influenced not only by drought but also by heat (STs2 = 35°C). However, LTc2 and LTs1 represent the thermal response of lines to a single stress, namely higher temperature and dehydration, respectively. Based on the highly significant correlation between these two variables, a cluster analysis of the 61 lines was then attempted using LTc2 and LTs1 as clustering variables. The resulting dendrogram clearly separated two groups of 30 and 31 lines (Fig. 2), lines in group 1 having higher LTc2 and lower LTs1 than lines in group 2. According to its thermographic behaviour, the reference line Bd21 would be included in group 1 (Fig. 3).

Correlation analyses between the climatic data of the 32 locations represented in the *B. distachyon* lines under study indicated that all of the temperature variables were correlated ($r=0.77$–0.99, $P<0.001$). Monthly precipitation values from January to April were also correlated ($r=0.79$–0.96, $P<0.001$). Monthly temperatures and precipitation were unrelated or poorly related, except for PREC_5 and PREC_6,

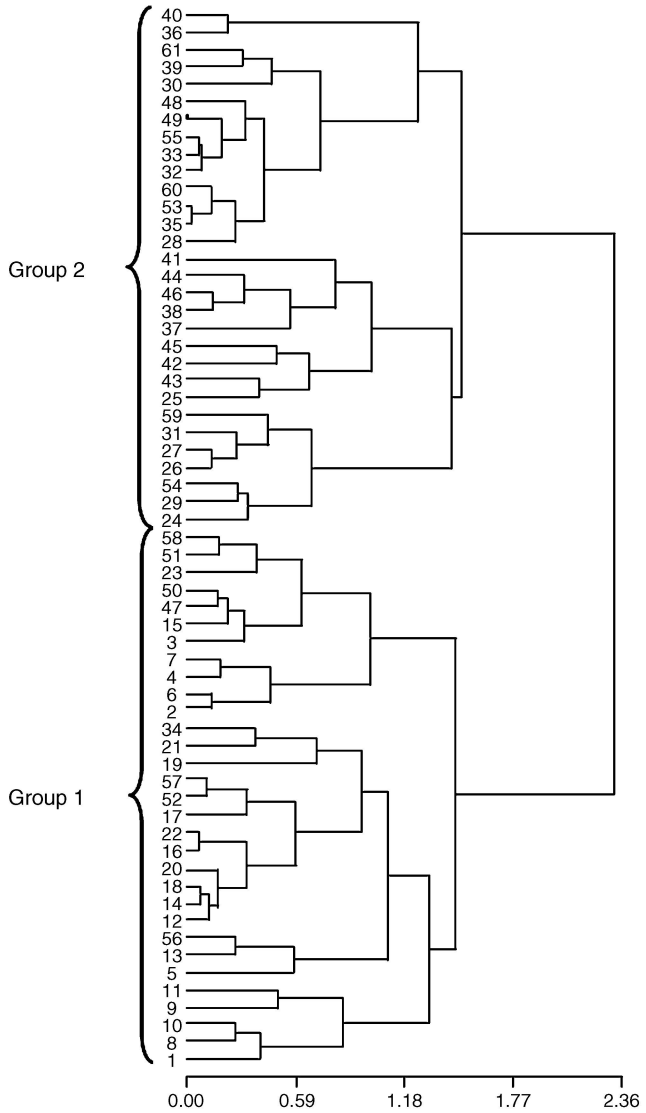

Fig. 2. Dendrogram obtained by cluster analysis based on Euclidean distance performed on the LTc2 and LTs1 values of the 61 *Brachypodium distachyon* lines.

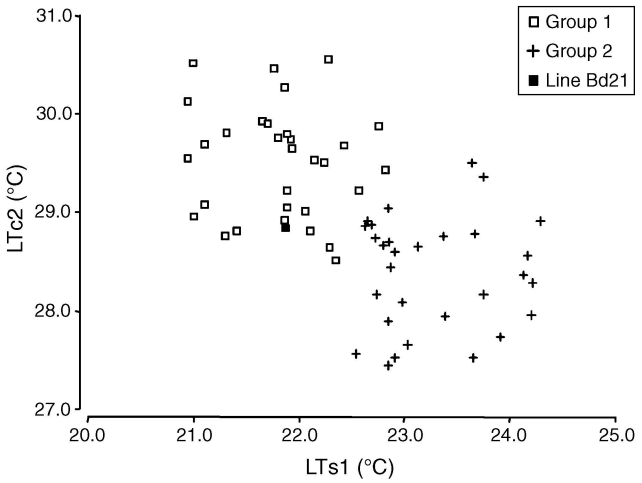

Fig. 3. Scatter plot of LTs1 and LTc2 for the 61 *Brachypodium distachyon* lines according to their grouping in the cluster analysis. The position of line Bd21 is noted.

which were highly negatively correlated with the temperature records of the site ($r = -0.41$ to -0.73, $P < 0.001$).

Results of the ANOVA indicated a significant influence of the collecting site on all LT variables (see Table 2). The climatic variables that most affected each of the thermographic parameters were identified through regression analyses, with the thermographic variables used as dependent variables and climatic variables as independent variables. Among all of the variables having a high statistical significance ($P < 0.01$), those included in the simple regression results shown in Table 4 are the bioclimatic, monthly precipitation and monthly temperature variables, which described the largest proportion of variance. These analyses indicated that 'mean temperature of the wettest quarter' (TMEA_WET_Q) was the variable that better explained differences in LTc2 and LTs1, accounting for 28% and 52% of their respective variation. Lines from locations with higher TMEA_WET_Q had lower LTc2 and higher LTs1 values. Variation for TD1 and TD2 showed relations with single climatic variables similar to LTs1, although the adjusted models fitted less well for TD2. Precipitation seasonality (PREC_SEA), directly related to the irregular distribution of rainfall periods during the year, had the highest significance ($P = 0.002$) and explained 15% of differences in LTc1. This thermographic variable measured the LT of control (irrigated) plants that were grown in cool conditions, and hence not affected by any apparent environmental stress. Multiple regression analyses showed that 24–33% of the variation of LTc2, LTs1, TD1 and TD2 was explained by the combination of PREC_2 and TMAX_4 or TMAX_3, with precipitation and temperature variables exhibiting opposite effects. Lower PREC_2 and higher maximum spring temperature decreased LTc2, and increased LTs1, TD1 and TD2, in the lines analysed. The precipitation had a higher individual effect than the temperature, except for TD2, where the variables exhibited similar effects ($R^2 = 0.12$ and 0.13, respectively). LTs2 was the only thermographic parameter not affected by any precipitation variable, 18% of its variation being explained by the combination of mean diurnal temperature range (TMEA_DIU_R) and TMAX_3.

The two groups of lines separated in the cluster analysis by LTc2 and LTs1 values (Figs 2 and 3) were compared for the thermographic and climatic variables (Table 5). The lines in group 2 showed significantly higher mean leaf temperatures under either medium or extreme drought (LTs1 and LTs2 values), and greater thermal differences between stressed and control plants (TD1 and TD2), whereas mean values of LTc2 were significantly lower than in group 1 lines. No differences were found between the two groups for mean LT of control plants under cooler greenhouse conditions (LTc1: 20.66 *v.* 20.27). Group 2 genotypes correspond to drier zones, with a mean annual precipitation of 582 mm, whereas the average annual precipitation of lines in Group 1 was 690 mm. Records for other rainfall variables, including total precipitation in the wettest quarter or month, were significantly lower in group 2 than group 1 lines (Table 5). The locations

Table 4. Results of multiple and simple linear regression analysis

Only the variables that generated the best multiple regression models are shown. The sign of the independent variable in the model is indicated in parentheses. For description of climate and thermographic variables, see Tables 1 and 2, respectively

Dependent variable	Independent variable	R^2	R^2 adjusted	s.d.	*P*-level
		Simple regression			
LTc1	PREC_SEA (+)	0.15	0.14	0.110	0.002
LTc2	TMEA_WET_Q (–)	0.29	0.28	0.020	<0.001
	PREC_2 (+)	0.18	0.16	0.003	<0.001
	TMAX_3 (–)	0.08	0.07	0.005	0.025
LTs1	TMEA_WET_Q (+)	0.52	0.51	0.020	<0.001
	PREC_2 (–)	0.23	0.22	0.004	<0.001
	TMAX_4 (+)	0.12	0.10	0.050	0.006
LTs2	TMEA_DAI_R (+)	0.12	0.10	0.100	0.007
	TMAX_3 (+)	0.09	0.08	0.040	0.017
TD1	TMEA_WET_Q (+)	0.49	0.48	0.030	<0.001
	PREC_2 (–)	0.21	0.20	0.010	<0.001
	TMAX_4 (+)	0.11	0.10	0.090	0.008
TD2	TMEA_WET_Q (+)	0.25	0.24	0.030	<0.001
	PREC_2 (–)	0.12	0.10	0.010	0.007
	TMAX_3 (+)	0.13	0.12	0.010	0.004
		Multiple regression			
LTc2	PREC_2 (+), TMAX_3 (–)	0.25	0.23	0.698	<0.001
LTs1	PREC_2 (–), TMAX_4 (+)	0.33	0.31	0.761	<0.001
LTs2	TMEA_DAI_R (+),TMAX_3 (+)	0.18	0.15	0.648	0.004
TD1	PREC_2 (–), TMAX_4 (+)	0.30	0.28	1.262	<0.001
TD2	PREC_2 (–), TMAX_3 (+)	0.24	0.22	1.119	<0.001

Table 5. Comparisons by *t*-test of the thermographic and climatic records between lines in groups 1 and 2 of the cluster analysis

Only the climatic variables that resulted significantly different are shown. For description of climate and thermographic variables, see Tables 1 and 2, respectively

Variable	Group 1 mean	Group 2 mean	*t*-test	*P*-value
	Thermographic variables			
LTc1	20.66	20.27	1.64	0.107
LTc2	29.51	28.39	7.73	<0.001
LTs1	21.83	23.27	–10.21	<0.001
LTs2	29.80	30.52	–4.70	<0.001
TD1	1.20	3.02	–5.99	<0.001
TD2	0.28	2.14	–8.51	<0.001
	Climatic variables			
TMEA_WET_Q	6.15	11.64	–6.50	<0.001
TMEA_DAI_R	12.68	13.15	–2.21	0.032
TMAX_3	13.14	14.18	–2.01	0.049
TMAX_4	15.07	16.24	–2.22	0.030
PREC_WET_Q	236.90	164.77	4.28	<0.001
PREC_WET_M	86.48	60.10	4.13	<0.001
PREC_COL_Q	233.35	154.17	4.30	<0.001
PREC_ANN	689.77	581.63	2.92	0.005
PREC_SEA	3.49	2.84	2.54	0.014
PREC_1	73.16	48.80	4.10	<0.001
PREC_2	74.42	47.20	4.45	<0.001
PREC_3	64.58	51.17	2.42	0.019
PREC_4	68.16	58.33	2.49	0.016
LANG	58.49	47.34	2.86	0.007

represented by group 1 lines are characterised by significantly wetter winter and early spring seasons than those represented by group 2 lines (compare mean values for PREC_COL_Q and PREC_1 to PREC_4 in Table 5). The distribution of precipitation was therefore more regular (i.e. lower mean values of PREC_SEA) at locations represented by group 2, taking into account that summer is a dry season at any Spanish location. Between-group differences were either not found or were just slightly significant regarding annual or monthly temperature records. Only the mean temperature of the wettest quarter was significantly higher at the collection sites of lines in group 2. Areas where original populations of group 2 lines were found also had higher aridity.

Discussion

The present study showed that LT values, or indices based on LT changes under controlled conditions, have potential for identifying *B. distachyon* lines with tolerance to water stress. Thermal imaging provides an ideal approach for the recording of LT in a large number of individuals and allows information on the temperatures of all areas in a scene to be obtained simultaneously in one image. However, LT depends not only on stomatal conductance but also on environmental factors such as air temperature, air humidity, radiation and boundary layer conductance, which determine the leaf energy balance (Jones 1999). In order to maximise the potential of thermal imaging to monitor stomatal conductance or estimate the transpiration rate, reference surfaces such as a well-watered and a non-transpiring crop are commonly included in the images (e.g. Leinonen and Jones 2004). Reference surfaces have two main purposes: first, to facilitate automated elimination of pixels representing sky, soils or background surfaces; and second, to minimise the effect of environmental factors that may influence LT when the images to be compared are taken under changing conditions (Leinonen

and Jones 2004; Grant *et al.* 2007; Guilioni *et al.* 2008; Costa *et al.* 2013). In our study, thermal images of all samples were taken in a closed, shaded greenhouse, in about 1-h lapse for each thermography session. In addition, foliar temperatures have been estimated based on the records of selected pixels that strictly corresponded to leaf tissues. In most cases, it was verified by the clearly warmer temperature of leaves over soils but it was also checked by the simultaneous colour imaging in the few borderline cases. Therefore, the use of reference surfaces seemed not necessary, and changes in LT between lines, between treatments or between recording dates can be assumed largely to represent variations in stomatal conductance.

For most of the lines analysed, the LT estimated under water deficit (LTs1 and LTs2) was higher than the corresponding value under irrigated conditions (LTc1 and LTc2, respectively), in agreement with earlier thermography studies in various crops (e.g. Inagaki and Nachit 2008; Reynolds-Henne *et al.* 2010; Costa *et al.* 2012). This LT increment, which results from stomatal closure, reflects a common response to drought by which plants are able to maintain leaf water status by reducing evapotranspiration. Decreased stomatal aperture under the first stages of drought may improve intrinsic water-use efficiency with a positive impact on plant growth (Chaves *et al.* 2003; Costa *et al.* 2013). The main disadvantage of this strategy is that the reduced transpiration can cause excessive leaf heating, with photosynthesis being restricted by stomatal closure and biochemical and photochemical limitations (Martin and Ruiz-Torres 1992; Medrano *et al.* 2002; Chaves *et al.* 2003). When plants grow in the field, the adverse effects of drought can be alleviated by a greater root system, which increases water extraction from soil. This has been demonstrated for some drought-tolerant wheat varieties (Olivares-Villegas *et al.* 2007; Reynolds *et al.* 2007). However, in standard greenhouse or growing chamber conditions, plants usually have a limited root development, and stomatal adjustment is probably the main mechanism to avoid leaf dehydration. Under either medium or severe stress, the difference between maximum and minimum LTs found here was ~3°C (Table 2). This suggests a wide range of stomatal behaviour in response to drought stress in this collection of *Brachypodium* lines. A difference of 1–1.2°C has been reported in *Arabidopsis* between wild plants and mutants with a reduced ability to close stomata in response to water deficit (Merlot *et al.* 2002).

Thermal difference between medium drought-stressed plants and their irrigated controls (TD1 values) varied considerably between lines. Some of the lines behaved as very sensitive to water deficit, leaves being significantly warmer in the stressed plants than in controls. However, other lines seemed insensitive to drought, with TD1 values close to 0, or even lower in two cases. The between-line variation was less pronounced at the end of the experiment (TD2 values), but the significant correlation between TD1 and TD2 suggests that such differential sensitivity to drought is largely genotype-dependent, which is also supported by the ANOVA results in Table 2. Olivares-Villegas *et al.* (2007) showed that, under water deficit, canopy temperature was highly heritable in a wheat recombinant inbred line population derived from two parents that diverged in their sensitivity to drought. Because our experimental conditions imposed a much reduced root development, differences in root architecture can hardly explain the wide variation found between lines in response to water deficit. Genotypic variation related to stomatal-based drought responses provides the most like explanation; however, other mechanisms including variation in hydraulic conductance cannot be excluded.

Optimum temperature conditions for growing *Brachypodium* are ~20−22°C (e.g. Luo *et al.* 2011; Catalán *et al.* 2012). Harsant *et al.* (2013) demonstrated that temperatures of ≥28°C have a negative impact on vegetative and reproductive growth in this species. Therefore, it can be assumed that irrigated plants were under moderate heat stress when LTc2 values were recorded.

The between-line difference for LTc2 (Table 2) supports a remarkable phenotypic plasticity related to stomatal behaviour in response to heat in our *B. distachyon* collection. Some watered plants had leaves as cool as or even cooler than the wet soils, whereas others showed LT values up to 3°C warmer. Such variation is comparable to the reported by Pradhan *et al.* (2012) in a group of accessions belonging to five distinct *Aegilops* species when growing at temperatures 11°C above the optimum for these wild wheat relatives. Those authors also found a greater decline for some yield components (grain number and individual grain weight) for the species that could not keep their LT as cool as others under heat stress. For diverse crops including wheat, genotypes with greater capacity for leaf cooling and lower canopy temperature have been shown to have a higher yield in hot regions (Radin *et al.* 1994; Fischer *et al.* 1998; Costa *et al.* 2012).

Combined heat and drought alters the leaf response relative to that observed under a single abiotic limiting condition. In our experiment, the mean and range of TD between drought-stressed and watered plants recorded under warmer conditions (TD2 values) were smaller than in a cooler environment (TD1 values), despite an additional week of water withholding in the treated blocks. This is likely evidencing the antagonist effect of heat and drought on stomatal conductance and related physiological traits (Gupta *et al.* 2012). An interaction between ABA treatment (which induces water stress) and incubation temperature on stomatal behaviour has been reported in legumes (Reynolds-Henne *et al.* 2010). Increasing temperatures increased stomatal opening, but a very high temperature (47°C) was required to initiate stomatal opening in the presence of ABA, indicating a tendency to prioritise water relations.

The negative correlation between LTs1 and LTc2 values indicates that the lines with higher LT under water-restricted conditions had lower LT under warm temperature (Table 3). It supports the idea that some lines in our collection have a more effective stomatal-based control to face limiting abiotic conditions than other lines. Differences in the structure, size or density of stomata and/or in their opening–closing ability in response to heat and drought stress have been indicated to explain between-genotype variation related to transpiration behaviour (e.g. Reymond *et al.* 2003; Masle *et al.* 2005; Liu *et al.* 2011; Manzaneda *et al.* 2012). Whether any or several of these factors are responsible for the variations between lines described here requires further analyses that are outside the scope of the present report.

The lines used in the study were developed from specimens collected in the wild. Thus, they can be assumed to represent

genotypes adapted to the eco-geographical characteristics of the collection sites. The whole collection represents a wide range of climatic conditions, especially for rainfall regime (annual precipitation range 378–954 mm) and Lang aridity index (range 26.3–103.7), but also for temperature, with mean temperature of the warmest quarter ranging from 16.1°C to 24.9°C and of the coldest quarter from 0.8°C to 12.6°C. Linear regression analyses revealed the significant relationship between the thermographic behaviour of the lines in response to drought and warm experimental conditions and some climatic characteristics of the location where the corresponding genotypes were collected (Table 4). The mean temperature of wettest quarter (TMEA_WET_Q) has the strongest influence on the thermographic behaviour within this *Brachypodium* germplasm collection. It explains ~50% of the between-line variation for the temperature of leaves under water-deficit conditions (LTs1) and for the thermal parameter related to the efficient stomatal-mediated response to drought (TD1), as well as almost 30% of the differences found when ambient temperature was above the optimum (LTc2). Bioclimatic variables are considered biologically more meaningful to explain adaptive patterns than are monthly precipitation or temperature records because they address extreme or limiting environmental factors that may affect plant growth (Hijmans *et al.* 2005). In agreement with that, TMEA_WET_Q was a better predictor of the variation for those thermographic parameters than the most influencing monthly precipitation or temperature variables, either alone or combined. If only raw climatic variables are considered, the precipitation seems more relevant than the temperature to the thermographic response of the genotypes to drought or heat-limiting conditions.

The lines analysed clearly separated into two groups with significant differences for all of the thermographic variables estimated under restricted conditions (Figs 2 and 3, Table 5). Higher LTs1 and TD values recorded for lines in group 2 support that these genotypes had greater stomatal closure under water-limited conditions and drought sensitivity, whereas their lower LTc2 values indicate increased evaporative cooling when under warm conditions. Our analysis has further demonstrated that these groups discriminate two sets of genotypes originating from contrasting habitats with marked variations in some climatic variables. Lines in group 2 came from drier zones, with warmer late winter and spring and greater aridity (Table 5). The rainiest season of the year is winter for most lines in group 1 (27 of 31), whereas it is spring for most lines in group 2 (21 of 30). This explains the highly significant difference between groups for the mean temperature of the wettest quarter. Therefore, lines in group 2 represent genotypes usually facing the effect of drought combined with warmer temperatures during spring. No geographic (latitude–longitude) or altitudinal effects were detected in the separation of the groups, which is in agreement with earlier studies that used physiological parameters such as leaf water content and water-use efficiency to evaluate drought response in wide *Brachypodium* germplasm collections (Luo *et al.* 2011; Manzaneda *et al.* 2012). Luo *et al.* (2011) established a phenotype ranking (tolerant, moderate tolerant, susceptible and most susceptible) of 57 natural populations of *B. distachyon* according to their drought response as measured by leaf wilting, chlorophyll fluorescence and leaf water content, among others. Line Bd21 was classified as susceptible by those authors, which agrees with the low drought adaptation predicted from our thermographic analysis for this reference genotype (Fig. 3).

Brachypodium hybridum (2n = 4x = 30) is an allopolyploid annual species derived from *B. distachyon* and the diploid *B. stacei* (2n = 2x = 20) (Catalán *et al.* 2012). Manzaneda *et al.* (2012) found a strong positive association between environmental aridity of the collection sites and predominance of *B. hybridum* over *B. distachyon* in natural populations from the Iberian Peninsula. Their study further demonstrated that the polyploid accessions were significantly more efficient in water use under water-restricted growing conditions than the diploids. A preliminary thermographic study involving a small set of *B. hybridum* lines showed that these presented higher mean values of LTs and TD than *B. distachyon* lines, the between-species differences being highly significant with extreme water deficit (Benavente *et al.* 2013). Under high-temperature conditions, watered plants of *B. hybridum* lines maintained cooler leaves than those of *B. distachyon*. The lines of *B. hybridum* used in that analysis originated from sites that are drier and warmer than the sites represented in the *B. distachyon* collection (data not shown). All of the above information provides evidence for a consistent thermographic behaviour of *Brachypodium* genotypes better adapted to unfavourable climate regimes (i.e. *B. hybridum* compared with *B. distachyon*, and group 2 compared with group 1 *B. distachyon* lines), and supports a key role of stomatal regulation in drought tolerance for this model plant.

Our results confirm thermography as a simple and fast tool for the identification of drought-tolerant genotypes in *Brachypodium*. Several other studies support the idea that increased leaf heating can be used as a reliable criterion for a high-throughput, preliminary screening of genotypes better adapted to drought stress in wide collections and segregant populations of some of the main cereals. Inagaki and Nachit (2008) analysed the effect of increasing water stress on LT of two synthetic hexaploid wheat lines differing in their water-deficit tolerance and found that the tolerant genotype had a higher LT value. Havaux and Tardy (1999) and Ji *et al.* (2012) suggested more intense stomatal closure as the main cause of drought resistance in varieties of barley and rice showing good performance in dry environments, although LT was not measured. Also in agreement with the present results for *B. distachyon*, Zaharieva *et al.* (2001) demonstrated that, under more extreme field conditions, populations of the wheat wild relative *Aegilops geniculata* originating from drought- and heat-prone areas exhibited higher leaf temperature, associated with higher water-use efficiency and lower epidermal conductance and transpiration, than those from areas with mild climate. Similar conclusions have been reported for *Arabidopsis* after comparison of the physiological consequences of dehydration in accessions from habitats with contrasting rainfall regimes (McKay *et al.* 2008). Decreasing leaf chlorophyll content has been noted as the likely photoprotection mechanism to mitigate the damaging effect of leaf heating in wild grasses and cereal landraces adapted to semi-arid environments (Havaux and Tardy 1999; Zaharieva *et al.* 2001; Royo *et al.* 2014).

In addition to the different LT responses to experimental abiotic constraints noted above, our study has shown wide between-line differences for LT in control, irrigated plants under optimum temperature conditions (LTc1; see Table 2), a parameter reflecting the constitutive temperature of each line. By using thermography, Kusumi *et al.* (2012) detected a rice mutant with constitutive low LT phenotype and demonstrated that, under well-watered conditions, this genotype had higher stomatal conductance and photosynthetic rate than the wild type genotype. Low constitutive stomatal conductance has been noted as the main drought-avoidance mechanism in an *Arabidopsis* genotype adapted to a dry habitat (McKay *et al.* 2008). However, we have not found any clear significant trend for LTc1 variation between lines originating from sites with contrasting climatic conditions (Tables 4 and 5). Furthermore, the negative correlation between LTc1 and LTs1 suggests that lines having a lower constitutive stomatal conductance show a higher stomatal conductance under drought stress. It is then likely that stomatal-based drought-adaptive mechanisms in *Brachypodium* are not constitutive but related to a more efficient closing response to avoid dehydration. This reinforces the recent focus on *B. distachyon* over *Arabidopsis* to investigate the molecular basis and physiological pathways of drought response in temperate cereals (Bertolini *et al.* 2013; Verelst *et al.* 2013; Bayramov and Guliyev 2014). To gain access to all genomic tools developed so far in this emerging model species, those studies are being conducted on a single reference genotype, the sequenced line Bd21 (International Brachypodium Initiative 2010; Catalan *et al.* 2014). However, it should be remembered that this genotype is not among those showing a good adaptation to water stress (Luo *et al.* 2011; and results reported in the present study).

Breeding of cereal crops better adapted to drier climatic conditions will need, among other features, the characterisation of genes involved in stomatal regulation as well as a survey of plants with a more efficient stomatal-based response to abiotic stress conditions. With these aims in mind, infrared thermography seems a reliable technique for easy identification of valuable genotypes in segregant populations and large germplasm collections of *B. distachyon* and temperate cereals.

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